THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

Date: November 17, 1972

1. Name of Investigator(s): (include Title and Degrees)

B. Bhagat, Ph.D.
Professor of Physiology

2. Institution & Address:

Department of Physiology
St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, Missouri 63104

3. Short Title of Project:

Effect of Chronic Administration of Nicotine and Smoking on Brain Biogenic Amines

4. Proposed Starting Date:

January 1, 1973

5. Anticipated Duration of this Specific Study:

3 years

6. Brief Descripton of Objectives or Specific Aims:

See Proposal.

7. Give a Brief Statement of your Working Hypothesis:

See Proposal.

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See Proposal.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See Page 2A.

10. Additional Requirements:

None.

11. Biographical sketches of all principal and professional personnel (append)

See pages 2C through 2G

12. List of publications: (Five most recent as pertinent) (append)

See page 2R.

FACILITIES AVAILABLE

1. Space. Our laboratory and office (approximately 600 sq. ft.) is well equipped with all the standard facilities. In addition, a cold room, radioisotope room, animal operating room, and machine shop are also available.

2. Equipment. In addition to standard laboratory equipment, such as glassware and other apparatus, the following items are available for our use: Grass stimulator, spectrofluorometer (Aminco-Bowman), mechanical shaker, and International portable refrigerated centrifuge.

3. Animal Research Space. Ample animal and laboratory space is available in the new renovated Animal Care Facility at St. Louis University Medical Center to permit proper conduct of these studies. These quarters are under the direction of a veterinarian who quarantines and conditions animals prior to use in experiments.

4. Library. An excellent medical library supports the research service. It includes over 5,000 volumes and regularly subscribes to 189 scientific periodicals. An excellent interlibrary loan system with the four Universities and two medical societies in our area gives us ready reference material promptly. The Yalem Computer Center of St. Louis University is readily available for the processing of data and gives a priority to medical research.

12. List of publications: (Five most recent as pertinent)

1. B. Bhagat: Effect of chronic administration of nicotine on storage and synthesis of noradrenaline in rat brain. Br. J. Pharmac. 38: 86, 1970.

2. B. Bhagat: Influence of chronic administration of nicotine on the turnover and metabolism of noradrenaline in the rat brain. Psychopharmacologia (Berl.) 18: 325, 1970.

3. B. Bhagat and M.W. Rana: Effect of chronic administration of nicotine on the concentrations of adrenal enzymes involved in the synthesis and metabolism of adrenaline. Br. J. Pharmac. 43: 250, 1971.

4. B. Bhagat, T. Bayer and C. Lind: Effect of chronic administration of nicotine on drug induced hypnosis in mice. Psychopharmacologia (Berl.) 21: 287, 1971.

5. P. Chang, B. Bhagat and J.C. Taylor: Effect of chronic administration of nicotine on acetylcholinesterase activity in the hypothalamus and medulla of the rat brain. An Ultrastructural Study. Brain Res. (In press).

CURRICULUM VITAE

BUDH DEV BHAGAT, Ph.D.

Born: R

Education:

Ph.D., Pharmacology, (Faculty Medicine),
London University, R

Postdoctoral in Pharmacology,
University of Wisconsin Medical School, R

Postdoctoral in Pharmacology,
University of Minnesota Medical School, R

Faculty Appointments:

Assistant Professor, Department of Pharmacology,
Howard University Medical School, 1964-66

Assistant Professor, Department of Pharmacology,
New York Medical College, 1966-68

Associate Professor, Department of Physiology
Associate Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1968-71

Professor, Department of Physiology,
Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1971-

Major Research Interests:

Autonomic Nervous System, Neurotransmitter, Cardiovascular

Committee Appointment:

Member - Advisory Board for "Neurosciences Research", Academic Press

Publications:

Approximately 169 publications to date.

CURRICULUM VITAE

CHANDRA HANS MISRA, Ph.D.

Born: R

Education:

B.Sc., Physics
Lucknow University, R

M.Sc., Chemistry
Lucknow University, R

Ph.D., Chemistry
Lucknow University, R

Experience and Appointments:

Research Associate, Department of Physiology
St. Louis University School of Medicine, 1972-

Postdoctoral Fellow, Department of Biochemistry
St. Louis University School of Medicine, 1971-1972

Research Associate, Endocrinology Research
Veterans Administration Hospital, St. Louis, Mo. 1970-1971

Assistant Research Officer, Department of Pharmacology
K.G. Medical College, Lucknow, 1969-1970

Research Assistant, Department of Pharmacology
K.G. Medical College, Lucknow, 1965-1969

Professional Organizations:

President of Chemical Association,
Lucknow University, 1961-62

Member of Association of Physiologist and Pharmacologist of India,
1965-66

Member of Indian Pharmacological Society, 1968-

Member of International Society for Biochemical Pharmacology

Research Publications:

1. STUDIES ON COMPLEX COMPOUNDS I. Co-ordination Compounds of Mercury and Aromatic Amines. C.H. Misra, S.S. Parmar and S.N. Shukla, J. Inorg. Nucl. Chem. 28, 147 (1966).

2. STUDIES ON COMPLEX COMPOUNDS II. Co-ordination Compounds of Mercury and Disubstituted Anilines. C.H. Misra, S.S. Parmar and S.N. Shukla, J. Inorg. Nucl. Chem. 29, 2589, (1967).

3. STUDIES ON COMPLEX COMPOUNDS III. Copper and Mercury Complexes of 2-chloro-6-methylaniline and 5-chloro-2-methylaniline. C.H. Misra, S.S. Parmar and S.N. Shukla, Can. J. Chem. 45, 2459 (1967).

4. STUDIES ON COMPLEX COMPOUNDS IV. Copper Complex of Quinazolone Hydrozides. C.H. Misra, S.S. Parmar and R.C. Arora, Inorg. Nucl. Chem. Letters 3, 603 (1967).

5. STUDIES ON COMPLEX COMPOUNDS V. Co-ordination Compounds of Mercury and Pharmacologically Active Amines. C.H. Misra, S.S. Parmar and S.N. Shukla, Can. J. Chem. 46, 2485 (1968).

6. STUDIES ON COMPLEX COMPOUNDS VI. Copper Complexes with Pharmacologically Active Cyclohexylamine Derivatives. C.H. Misra, S.S. Parmar and J.P. Barthwal, Can. J. Chem. 47, 4705 (1969).

7. STUDIES ON COMPLEX COMPOUNDS VII. Copper Complexes of Thiosemicarbazones as Possible Anticancer and Antitubercular Compounds (Communicated). (In Press).

8. STUDIES ON COMPLEX COMPOUNDS VIII. Metal Complexes of Schiff's Bases as Antibacterial (Communicated). (In Press).

9. Evidence Towards Possible Stimulation of Catecholamine Biosynthesis by Copper-Tyrosine Complex. S.S. Parmar, J.P. Barthwal, K.P. Bhargava, and C.H. Misra. Fifth International Congress on Pharmacology, San Francisco.

10. Studies on the Activity of Enzymes in Thyroglobulin and Albumin Induced Immunity at Different Tissue Levels. (In Preparation).

11. Analytical Application of the Schiff's Base for Iron Estimation. (In Press).

12. STUDIES ON COMPLEX COMPOUNDS IX. Metal Chelates of Biguinides. (In Preparation).

13. Antibacterial and Antifungal Properties of Some New Mercury Complexes. (In Press).

CURRICULUM VITAE

YU-CHIANG LEE, Ph.D.

Born: R

Education:

B.S., Chemistry, Taiwan Christian College, R

Ph.D., Biochemistry, Oklahoma State University, R

Experience and Appointments:

Research Associate, Department of Physiology
St. Louis University School of Medicine, 1971-

Postdoctoral Fellow, Department of Biochemistry
The University of Texas Medical School at Dallas, 1969-1970

Research Assistant and Graduate Student, Department of Biochemistry
Oklahoma State University, 1964-1969

Laboratory Instructor, Department of Chemistry
Taiwan Christian College, 1960-1963

Professional Organizations:

Member, REDACTED

Member,

Member, R

Publications:

1. K. Yamaguchi, Y.C. Lee, and R.K. Gholson. Nicotinamide methyl transferase on the regulation of NAD biosynthesis. International Congress of Biochemistry IV-F-165 (1967).

2. Y.C. Lee, R.K. Gholson and Nicholas Racia. Identification of Two New Nicotinamide Metabolites in Germ Free Rats. A.C.S. National Meeting (1968) Abstract Biol. 24.

3. Y.C. Lee, R.K. Gholson and Nicholas Racia. Isolation and Identification of Two New Nicotinamide Metabolites. J. Biol. Chem. 244, 3277 (1969).

4. Y.C. Lee, R.M. McKenzie, R.K. Gholson and Nicholas Racia. A Comparative Study of the Metabolism of Nicotinamide and Nicotinic Acid in Normal and Germ Free Rats. Biochem. et Biophy. Acta 264, 59 (1972).

5. Taruna Dave, Y.C. Lee, R.J. Bryan, R.C. Srivastava and B. Bhagat. Activity of Enzyme Involved in Synthesis and Degradation of Norepinephrine in Guinea Pig Isolated Vas-Deferens During Intermittant Nerve Stimulation. Federation Abstracts 31, 543, 1972.

6. B. Bhagat, T. Dave, Y.C. Lee and R.J. Bryan. Nerve Stimulation and the Activity of Catecholamine Metabolizing Enzyme in Guinea Pig Isolated Vas-Deferens. Amer. J. Physiol. (in press)

13. Budget (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
B. Bhagat, Ph.D.	20%	REDACTED
C. H. Misra, Ph.D.	100%	
Y.-C. Lee, Ph.D.	100%	
Technical		
R. J. Bryan	100%	
Animal Caretaker	50%	
Fringe Benefits		
	Sub-Total	REDACTED
B. Consumable Supplies (list by categories)		
Radioactive material		1,000.
Drugs and chemicals		1,000.
Animals		2,500.
Maintenance of equipment		400.
	Sub-Total	4,900.
C. Other Expenses (itemize)		
Travel to attend National meetings		400.
Photographic material		200.
Reprint costs		200.
	Sub-Total	800.
D. Permanent Equipment (itemize)		
NONE		
E. Overhead (15% of A+B+C) (exclusive of fringe benefits)		4,605.
	Total	$36,805.

10035388798

11/27/70

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	4,900.	800.	--	4,605.	36,805.
Year 3		4,900.	800.	--	4,605.	36,805.

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature B. Bhagat
Director of Project

(314) 865-2288, x. 412
Telephone

Signature Y.-C. Lee
Business Officer of the Institution

Telephone

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Nicotine Behavioral Changes and Brain Biogenic Amines	National Institute of Mental Health	$24,947.	7/1/72 - 6/30/73

Pending

INTRODUCTION

Ever since smoking became established, it has enjoyed such striking success that there can be little doubt that the habit is rewarding to the participant. And since the discovery, more than a century ago, that the most powerful pharmacological agent in tobacco is nicotine, the obvious view has often been expressed that people who use tobacco are seeking the physiological responses to this alkaloid. Johnston (Lancet, 11:792, 1942) reported that the injection of nicotine resulted in a pleasant sensation to smokers, whereas nonsmokers reported an unpleasant sensation. And Deneau and Inoki (Ann. N.Y. Acad. Sci. 142:277-279, 1967) found that monkeys may even administer nicotine intravenously to themselves. These findings strongly suggest that nicotine is the active constituent of tobacco smoke and that the effects of nicotine from smoking can be imitated by intravenous injection of this alkaloid.

Recently Goldfarb et al (Psychopharmacologica 17:89, 1970) compared subjects' base line smoking rate with their own brands of cigarettes to their rates when smoking specially prepared lettuce cigarettes varying in nicotine content from zero to 2-25 mg nicotine per cigarette. They found that subjects do perceive differences in nicotine content of cigarettes. Armitage et al (Nature, 217, No. 5126:313, 1968) showed that small amounts of nicotine injected intravenously usually increased the lever pressing activity of rats and caused a change in the EEG of cats, indicative of cortical activation. These results in experimental animals are consistent with the subjective impressions of some smokers that inhalation of tobacco smoke causes them to be more alert and efficient. Thus, their findings suggests one good reason for the extraordinary fact that despite the widely publicized risks, many billions of cigarettes are used in this country alone every year. It appears that nicotine produces highly describable effects upon the brain. It seems likely that some people smoke in order to dose themselves with nicotine.

Drugs which produce behavioral changes in man and animals can also produce changes in brain catecholamine patterns. Neurochemical, pharmacological and behavioral studies have provided a number of findings compatible with this hypothesis. Reserpine, a hypotensive and tranquilizing agent which characteristically depletes the brain of norepinephrine and other amines, has been observed in a significant proportion of patients receiving it to result in a state closely resembling endogenous depression. On the other hand, a number of drugs which elevate mood and have been found of value in the treatment of depression appear to act on central norepinephrine in ways which could increase its physiologically active concentration, either by inhibiting the enzyme responsible for its presynaptic deamination, by favoring its release or by inhibiting its reuptake, presumably at the central synapse (Kety et al, Proc. N.A.S. 58:1249, 1967).

Recently, tricyclic antidepressant drugs have been shown to increase the rate of synthesis of brain norepinephrine (Neff and Costa, 1967). The therapeutic efficacy of electroconvulsive shock in depression had been explained in its ability to induce acceleration in the synthesis of norepinephrine. Ryo Takahashi et al (1968), presented evidence that indicates that overproduction

of catecholamines might occur in the patient during the manic state and underproduction during the depressive state.

The catecholamines (norepinephrine, epinephrine and dopamine) and indoleamine (serotonin) are the most important brain amines. Norepinephrine is present in various regions of the mammalian brain, particularly in the hypothalamus. Epinephrine also occurs in the brain in high concentrations in that area. Highest concentrations of dopamine are found in the basal ganglia; lower concentrations of this amine are found in most other brain areas. Serotonin is present in appreciable amounts in the brain and its distribution is similar to that of epinephrine and norepinephrine.

It has been suggested that nicotine might produce some of its actions in the central nervous system by the release of norepinephrine or other amines from stores in central nervous system tissue (Vogt, Proc. 4th Int. Cong. Biochem., F. Brucker, Ed., 3:279). Several workers (Hansson et al, Arch. int. Pharmacodyn. 1964:148, 153; Westfall and Watts, J. Neurochem. 1964: 11, 397) measured catecholamines in the brain after single or repeated injections of nicotine, but failed to observe any clear-cut changes in the norepinephrine content, although there was some indication of change in serotonin content. Recently, Westfall et al (Ann. N.Y. Acad. Sci. 1967: 142, 83) determined the effect of nicotine on subcellular distribution of catecholamines, and showed that no significant effect on the N.E. content was observed after 0.5 mg and 1 mg/kg nicotine i.p. in whole mouse brain, and of rat diencephalon while a significant decrease in dopamine content of the brain was observed. These observations did not give clear conclusive results. The next logical step was taken in this laboratory where, with the help of labelled norepinephrine, it was shown (Bhagat, B., Kramer, S.Z., and Seifter, J., Europ. J. Pharmac. 2:234-235, 1967) that even very small doses of nicotine caused an increased release of ^{3}H-norepinephrine from the brain. Assuming that the release of ^{3}H-norepinephrine reflects an accompanying released endogenous norepinephrine then the failure to find a decrease in the concentration of cerebral norepinephrine must, in turn, reflect a rate of replenishment equal to the rate of release.

Later, in 1970 in this laboratory, the effect of chronic administration of nicotine on catecholamine concentration in the rat brain was determined (Bhagat, Br. J. Pharmac. 38:86-92, 1970). Nicotine was administered to rats 5 times a day for 6 weeks. When calculated in terms of body weight this is equivalent to 3 packs of cigarettes a day. It was found that following the chronic administration of nicotine the endogenous levels of neurohormones in the brain were unaltered, but that there was a clear increase in both the synthesis and the utilization of these neurohormones. These results were true for norepinephrine, dopamine, acetylcholine and 5-hydroxy-tryptamine. The important conclusion was obvious. Endogenous levels of neurohormones in brain tissue are deceptive indicators of the responses of the tissues to stress; instead, the important factor in the response of neurohormones to nicotine is their rate of turnover which provides a yardstick of central nervous sympathetic activity. These observations suggest that chronic administration of nicotine causes adaptive changes in the body so that there is a sustained rise in the rate of synthesis of norepinephrine to meet the requirement for norepinephrine in the face of continued enhanced utilization.

This may also help to explain the increase in the lever pressing activity of rats given nicotine frequently (Armitage et al, 1968) and the improvement in the learning ability of rats and mice in several different tests after low subcutaneous doses of nicotine (Bovet et al, 1967).

Yet, while the rates of synthesis and utilization were increased by repeated administration of nicotine, endogenous levels continued to remain stable. It is suggested that alterations in the turnover rates rather than levels may be a correlated of nicotine induced behavioral changes. Norepinephrine is in a dynamic state of release, metabolism and biosynthesis, yet despite these changes the absolute levels of tissue norepinephrine remains remarkably constant. In order to understand the effect of smoking or chronic administration of nicotine on the catecholamine pattern and to obtain more meaningful data it is essential to study all the aspects of synthesis, storage, disposition and metabolism of catecholamine.

Only then can we obtain useful information which might alter or enhance our knowledge about the effects of smoking on the central nervous system.

AIMS

In our proposed study, animals will be exposed to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure. Other animals will be treated with nicotine or cotinine, a metabolite of nicotine. We will examine brain and cardiovascular tissue for changes in the pattern of catecholamine, 5-hydroxytryptamine, and acetylcholine forthe following reasons:

1. a) To determine the manner in which smoking affects the brain particularly its chemistry.

   b) To determine whether the changes in the chemistry of brain induced by smoking are identical to that caused by nicotine.

   c) To determine whether nicotine-induced alterations are due to nicotine per se or mediated through its metabolite cotinine.

2. To develop a more detailed understanding of short and long term time-courses in the altered rate of synthesis and utilization of neurohormones in the central nervous system and cardiovascular tissues. We will examine animals at specific times following the start of smoking or chronic treatment with nicotine or cotinine and once the maximum changes have developed, during the subsequent period of withdrawal of the drug. An understanding of these factors is essential to attempt to define the mechanism involved in the synthesis and metabolism of these neurohormones.

3. To examine the effect of smoking, nicotine or cotinine on certain behavior and to correlate the changes in acquired behavior with changes in the pattern of neurohormones.

4. To try to define the mechanism involved in the changes in the pattern of neurohormones after smoking or nicotine by modifying or preventing those changes by pharmacological agents.

EXPERIMENTAL PROCEDURE

1. Preparation of Animals

Rats (Holtzman strain) weighing about 60 to 70 gm will be used throughout this study. Animals will be placed in cages which will be kept under similar conditions of lighting and humidity in a room maintained at a temperature within the range of 21.0 $\pm$ 0.5°C. Food and water will be supplied ad libitum. No more than 6 rats (unless otherwise required) will be housed in each cage, since it was observed that crowding of animals increased the tyrosine hydroxylase by 32%. All animals will be acclimatized to the new environment for a period of one week before they are subjected to any treatment.

2. Body weight

Body weight will be recorded weekly.

3. Food and Water

Food and water intake will be measured daily and expressed per 100 gm of body weight.

4. Measurement of Systolic Blood Pressure

The systolic blood pressure will be measured weekly in unanesthetized animals using a pulse transducer applied to the tail.

5. Sex Difference

Whether there is a sex difference in the effect of smoking or nicotine, experiments in the females will be compared with males. Some experiments will be performed on pregnant rats.

6. Chronic Treatment of Nicotine or Cotinine

Nicotine or cotinine will be injected in various doses ranging from 0.05 to 1 mg/kg. Each dose will be injected subcutaneously, 3 times a day for at least 10 weeks. Control animals will be injected with equivalent amounts of saline. Animals will be used for study 12 hours after the last injection of nicotine.

7. Exposure to Smoke

Animals will be conditioned for at least one week prior to smoke exposure. Rats will be inserted into the animal cone holder and placed on the operating

machine without cigarettes, three times each day for 10 minutes. Suitably conditioned animals will enter the cone holders voluntarily.

Animals losing weight generally more than one gram per day during the conditioning period will be discarded, since these animals will not survive a chronic exposure. Following one week's exposure without smoke rats will be adapted with smoke to cigarette-concentration smoke for 8 minute exposure, 3 times a day.

The Walton Horizon Smoke Exposure Machine (developed under contract by the Council for Tobacco Research, U.S.A.) will be used. It has a capacity to expose 12 young rats to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure.

Essentially smoke will be produced by "positive" puffing (blowing) metered air through a horizontally held cigarette enclosing in a plastic dome during a timed two second puff. The two second puff interval is defined as the interval when the dome is in contact with the cigarette holder plate. The average puff volume's defined as the average puff volume of smoke produced during the first eight puffs. The 35 ml is the average puff volume of smoke produced during the first eight puffs.

In the normal one minute cycle of operation the two second puff will be followed by a 15 sec hold period, i.e., for a total exposure time of 17 sec. This will be followed by a thirty sec. purge period to sweep out the smoke and a 13 sec rest period. The smoke will be pushed into a constant volume (384 cc) smoke exposure chamber. Uniform mixing will be achieved with a mechanical mixer attached to one of the animal cone holder plates.

Animals(conditioned for at least one week prior to smoke exposure) will be held in cone shaped holders and will breathe the exposure chamber contents with their noses just inside the smoke chamber. They will be removed from the cone holder promptly after exposure to avoid water loss due to sweating and the additional stress of excessive confinement.

Cigarettes

Kentucky reference cigarettes (IRI) with different levels of nicotine will be used. They will be equilibrated for at least 24 hr at 76 (±2)°F to to (±2)% relative humidity atmosphere, by placing them unwrapped, with package opened into a dissicator (on wire mesh shelves) containing a 74% w/w glycerol-water solution in the bottom compartment. The cigarettes will be placed losely into the chamber.

8. Behavioral Studies

A) Spontaneous motor activity. It will be measured for 15 min periods in doughnut-shaped cages 12 inches in diameter with 3 inch wide circular runways. Four equally-spaced 1 inch panels in the floor of each cage activate microswitches connected to an electromechanical counter. Measurement will be conducted in a ventilated, sound-insulated box containing four activity cages.

For consecutive daily measurements, animals will be always placed in the same cage. Before nicotine treatment will begin, all rats will be subjected to six or seven daily 15 min sessions during which they will develop an accomodation.

B) Rotarod Performance will be measured by placing the rats on a cylinder about 5 inches in diameter and 6 inches long, which will be rotated eight revolutions per min by an electric motor. Those animals that learned to walk the rod for a period of at least 3 minutes will be given treatment.

C) Conditioned avoidance behavior will be examined by a shuttle-box technique (Rech, J. Pharmacol. exp. Ther. 146: 369, 1964). Each avoidance trial will be initiated by the conditioned stimulus, a small light activated on the side of the cage occupied by the rat. After 5 seconds the grid floor on that side of the cage will be electrified for an additional 5 sec, after which both the light and shock will be terminated together. If the rat moved to the unlighted side during the initial 5 sec, the response is scored as an avoidance. The trial will be repeated every 30 sec and 20 inch trials constituted a test session. Only rats which will average more than 15 out of 20 avoidance responses after training will be used in this study and will be given nicotine treatment.

9. Catecholamines

All aspects of catecholamines pattern (synthesis, storage, release, uptake, disposition and catecholamine enzyme - tyrosine hydroxylase (TH), monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT)) will be determined.

a) Brain

The metabolism of central monoamines is studied after labeling of central aminergic neurons by injection of microquantities of radioactive amines or their precursor in the cerebrospinal fluid. Changes in synthesis or utilization of radioactive amines can be estimated by measuring the activity of the amines in the various structures of the brain as a function of the time. In vivo release of monoamines can be estimated by superperfusing a local area of the brain rich in aminergic terminals and thus by collecting radioactive amines previously synthesized from their labelled precursor.

The brain of animals at various intervals of treatment will be studied for the following parameters: 1) endogenous norepinephrine, 2) its capacity to uptake and accumulate ^{3}H-norepinephrine, 3) the rate of metabolism of ^{3}H-norepinephrine, 4) the rate of conversion of ^{3}H-tyrosine to ^{3}H-norepinephrine, 5) monoamine oxidase activity, 6) catechol-o-methyl transferase activity, 7) tyrosine hydroxylase activity, 8) disposition of dopamine, 9) rate of conversion of ^{3}H-choline to ^{3}H-acetylcholine, and 10) rate of conversion of ^{14}C-tryptophan to ^{14}C-5-HT. In order to gain more information, the brain will be divided into various parts (telencephalon, hypothalamus, cerebellum, pineal body, colliculii, pons, medulla) and in each of these parts all the parameters will be measured.

b) Cardiovascular tissue

Norepinephrine in the tissue innervated with sympathetic nerve endings is inactivated by at least three mechanisms: a) uptake and storage in nerve terminals, b) o-methylation by catechol-o-methyl transferase (COMT) and c)oxidative deamination by monoamine amine oxidase (MAO). Inactivation by uptake of norepinephrine is more important than inactivation by metabolism. In support of this is the observation that physiological effects of injected norepinephrine are rapidly terminated, even after both MAO and COMT are inhibited.

In the heart and other sympathetically innervated organs, adrenergic nerve terminals are distributed throughout the entire tissue, whereas in the blood vessels, adrenergic nerve terminals are confined only to the adventitia and the underlying portions of the media. For this reason, catecholamines are inactivated differently in the vascular smooth muscle. In the adventitia and underlying portion of the media, like any other organ, the inactivation by binding seems to predominate over inactivation by enzymatic destruction, whereas in the greater part of the media norepinephrine is primarily activated not by uptake but by enzymatic breakdown. Cocaine, which is known to block the uptake of norepinephrine and thereby causes supersensitivity of the organ to norepinephrine, confines its potentiating action to the adventitia only, but does not affect the uptake of norepinephrine in the media.

Recently Berkowitz et al (J. Pharmac. exp. Ther. 177:119, 1971) have shown an uneven regional distribution in the blood vessels: the distal portions of the aorta and mesenteric artery had twice the content of the proximal tissue. Blood vessels take up less than 20% as much norepinephrine ^{3}H from the circulation as the heart.

Since smoking is implicated in the development of cardiovascular diseases and particularly to death from coronary heart disease, it is therefore necessary to determine the effect of smoking or chronic administration of nicotine on the synthesis and disposition of norepinephrine in the cardiovascular tissues. The following tissues will be examined: adrenal gland, superior cervical ganglia, heart, aorta, superior mesenteric artery, renal arteries, abdominal (inferior) vena cava and mesenteric vein. Changes in catecholamine pattern will be determined at various intervals following treatment and following withdrawal. The following parameters will be measured in the adrenal gland: 1) norepinephrine, 2) epinephrine, 3) TH activity, 4) MAO activity, 5) COMT activity, 6) phenyl-ethanol-N-methyl transferase.

The following parameters will be measured in superior cervical ganglia: 1) norepinephrine, 2) TH activity, 3) MAO activity, 4) COMT activity.

The following parameters will be measured in heart and vascular tissues: 1) norepinephrine, 2) capacity to take up and accumulate ^{3}H-norepinephrine, 3) the rate of metabolism of ^{3}H-norepinephrine, 4) rate of conversion of ^{3}H-tyrosine to ^{3}H-norepinephrine, 5)MAO activity, 6)COMT activity, 7) TH activity.

All vascular tissues will be carefully cleaned of adhering tissue with forceps or a small nylon brush as described by Koletsky et al (Proc. Soc. Exp.

Biol. Med. 102: 12-15, 1959). Microscopic examination of the vessels will be made to confirm that adhering tissues (connective tissue, fat and extravascular nerves) have been removed.

METHODS

Animals will be killed by a blow on the head and decapitated. Various tissues will be rapidly removed, cleaned, frozen on dry ice and stored at 20°C prior to analysis.

Chemical Methods

1. Endogenous norepinephrine will be assayed by the method of Anton and Sayre (J.P.E.T. 133:360, 1962). The method involves the selective absorption of catecholamines onto a constant amount of aluminum oxide, elution with a constant volume of perchloric acid and their measurement by the formation of fluorescent trihydroxyindole in the presence of potassium ferricyanide and alkaline ascorbare. To differentiate between epinephrine and norepinephrine, fluorescence is measured at 2 different pHs (pH 2-3 and pH 5-7). In the lower pH range, norepinephrine compared to epinephrine has a negligible fluorescence. Of the naturally occurring analogues of norepinephrine, only dopamine interferes but this interference is reported to be relatively small. Samples will be run in duplicate and recovery rates of standard amounts of epinephrine and norepinephrine are calculated for each analytical run. Recoveries up to at least 75% from biological materials have been reported.

2. ^{3}H-norepinephrine will be estimated by adding an aliquot of eluate (obtained after the alumina absorption of labelled amine as described above) in the counting solution (Instagel: Packard Instrument Co.) and the radioactivity will be determined in a Nuclear Chicago Scintillation counter.

3. ^{3}H-catechol deaminated metabolites will be assayed by the method of Kopin et al (J. Biol. Chem. 236: 2109, 1961).

4. ^{3}H-normetanephrine will be assayed by the method of Iversen et al (J.P. E.T. 150:173, 1965).

5. ^{3}H-methylated deaminated metabolites will be estimated by the difference between the total radioactivity of the tissue extracts and the sum of other metabolites.

6. Serotonin and dopamine will be simultaneously measured along with norepinephrine according to the method of Fleming et al (Analytical Chem. 37:629, 1965).

Enzyme Studies

Tissue will be removed, cleaned, weighed and homogenized in 2.0 ml of ice cold .25M sucrose. An aliquot (10 ul) of the homogenate will be used for assay of monoamine oxidase activity. The remaining homogenate will be centri-

fuged at 26000 g for 20 min. Aliquots of the clear supernatant fluid will be assayed for tyrosine hydroxylase, PNMT and COMT activities.

Monoamine oxidase activity will be assayed by measuring the conversion of ^{14}C-tryptamine to ^{14}C-indoleacetic acid as described by Wurtman and Axelrod (Biochem. Pharmacol. 12:1439, 1964).

Catechol-o-methyl transferase (COMT) will be assayed by measuring the formation of ^{14}C-metanephrine on incubation with (-) epinephrine and ^{14}C-methyl-s-adenosylmethonine as described by Axelrod (in Methods of Enzymology, Vol. 5, p. 748, 1959, New York Acad. Press).

Tyrosine hydroxylase activity will be assayed by the method of Levitt et al (J.P.E.T. 148:1, 1965) with modifications described by Mueller et al (J.P.E.T. 101:379, 1969).

Phenylethanol-N-methyl transferase activity will be assayed by the method of Axelrod (J. Biol. Chem. 237:1657, 1962) using normetanephrine as the substrate and ^{14}C-S-adenosylmethionine will serve as a methyl donor.

Synthesis of Acetylcholine

Synthesis of acetylcholine will be measured from the rate of conversion of ^{3}H-choline to ^{3}H-choline to ^{3}H-acetylcholine according to the method by Marchbank (Biochem Pharmacol. 18:1763-1766, 1969).

Synthesis of 5-hydroxytryptamine

The measurement of 5-hydroxytryptamine turnover rate in the rat brain from the conversion of ^{14}C-tryptophan to ^{14}C-5-hydroxytryptamine will be made according to the method by Lin et al (J. Pharmacol. exp. Ther. 179:232-238, 1969).

Synthesis of Norepinephrine in Isolated Tissues

The measurement of norepinephrine turnover rate will be made by the amount of ^{3}H-norepinephrine formed from the ^{3}H-tyrosine according to the method of Weiner and Rabadjija (J. Pharmacol. Exp. Ther. 160:61-71, 1968). Many of these methods are already operative in our laboratory. The others will be set up for the purposes of this investigation.

SIGNIFICANCE OF RESEARCH

The importance of careful base-line observations is an appropriate model for understanding the mechanism of action of nicotine and smoking on the central nervous system and cardiovascular system cannot be overemphasized. Only in this way can we distinguish between early and late changes, between primary and secondary effects and between fundamental and the incidental. The investigation proposes to study the effect of nicotine and smoking on the pattern of catecholamines, 5-hydroxytryptamine and acetylcholine and to correlate these changes

with acquired behavior. Furthermore, we propose to determine the changes in the pattern of neurohormones and in the acquired behavior associated with withdrawal of nicotine or cessation of smoking.

It is the ambitious long-term aim of this project to work toward the achievement of such a breakthrough, or at least make a significant advance in understanding the effect of smoking on the central nervous system and in the cardiovascular system. We are more than hopeful that our efforts will aid in the elucidation of the mechanism of the action of nicotine on the central nervous system and thereby answer the question "Why do we smoke?"

Cigarette smoking has been implicated by epidemiological studies as one of the "major hazards to health in the United States." Not only has it been associated with respiratory diseases and disorders, but it has also been implicated in the development of cardiovascular diseases, particularly in cases of death due to coronary diseases. So far, no causal mechanism has been found to explain these statistical relationships. It is our belief that these studies promise to throw light on the mechanism of action of nicotine and smoking and may thus assist in the development of prophylactic measures and help place therapy on a more logical basis in the treatment of cardiovascular diseases. We are thinking in particular of patients with coronary heart disease.